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Molecular Cloning and Relationship to Freezing Tolerance of Cold-Acclimation-Specific Genes of Alfalfa[1]

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ABSTRACT

Cold-acclimation-specific (CAS) gene expression has been examined by screening a cDNA library prepared from poly(A)$^+$ RNA of cold-acclimated seedlings of a freezing-tolerant variety of alfalfa (*Medicago falcata* cv Anik). Three CAS cDNA clones, pSM784, pSM2201, and pSM2358, representing different sequence species, have been used to investigate the relative abundance and time-course of accumulation of corresponding transcripts. Results obtained show that the expression of these CAS genes is regulated in a coordinated manner most likely at the level of transcription. The expression of genes, as measured by mRNA abundance corresponding to the three CAS cDNA clones, is not stimulated or induced by heat shock, water stress, abscisic acid, or wounding. A positive correlation is observed between the expression of these cloned sequences and the degree of freezing-tolerance in four alfalfa cultivars.

Freezing temperatures constitute one of the most important environmental constraints limiting the productivity and distribution of plants. Although plants are known to differ in their ability to withstand freezing temperatures, the molecular/genetic basis of this differential freezing-tolerance is unclear. It is known, however, that a prior exposure of plants to low nonfreezing temperatures (cold-acclimation) increases their tolerance to subsequent freezing (14). Many physiological and biochemical changes are known to occur in plants during cold-acclimation (5, 10, 13, 21) and it has been suggested (26) that these changes are mediated by altered gene expression. Although changes in patterns of protein synthesis during cold-acclimation have been observed (2, 8, 9, 17, 18) it is not known at what level of gene expression such changes are regulated.

We previously reported changes in protein and mRNA profiles of an overwintering, freezing-tolerant alfalfa cultivar (*Medicago falcata* cv Anik) (17) and of a relatively freezing-sensitive cultivar, Saranac (*Medicago sativa* L.) (18). Although both cultivars show changes in protein profiles during acclimation, the profiles vary with each cultivar. In order to further investigate the molecular genetic basis of freezing tolerance, we have examined the specificity and accumulation of mRNAs by differential screening of a cDNA library. Here we report the isolation of cDNA clones of genes which are specifically expressed during cold-acclimation. Furthermore, the level of expression of these genes as measured by mRNA abundance in several cultivars of alfalfa is positively correlated with the varietal level of freezing-tolerance.

MATERIALS AND METHODS

Plant Material, Cold-Acclimation and Freezing-Tolerance

The major experimental plant material used in this study was seedlings of alfalfa (*Medicago falcata* cv Anik). In order to compare gene expression and freezing-tolerance among different cultivars, three other cultivars with varying freezing-tolerance were used. These are cultivars Iroquois (*Medicago media*), Algonquin (*M. media*), and Trek (*Medicago sativa*). All four cultivars show different levels of freezing-tolerance, Anik showing the highest and Trek the lowest (11). Seedlings of all cultivars were grown as described previously for Anik (17).

Cold-acclimation at 4°C was carried out as described (17) for the time periods mentioned in the text or figure legends. Tests of freezing tolerance were also carried out as described previously (17) except that several temperatures, namely, −5°C, −8°C, −12°C, and −15°C were used and LT_{50} values[4] were determined.

Administration of Stress

Seedlings were subjected to water stress, heat shock, wounding, and ABA treatment. ABA was used because it has been implicated in plant responses to environmental stresses (14), particularly to water stress (14) and low temperature stress (1, 3). Water stress was imposed by placing the seedlings in polyethylene glycol-6000 (water potential of −15 bars), heat

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[4] Abbreviations: LT_{50}, temperature at which 50% seedlings fail to survive; CAS, cold-acclimation-specific; ds, double-stranded; kb, kilobase; ss, single-stranded; SSC, 150 mM NaCl-15 mM sodium citrate.

shock was administered by exposure to 42°C for 3 h, wounding was administered by cutting the seedlings into small segments and placing them in water at 25°C for 3 h, and abscisic acid was used at 75 μM with seedlings placed in the solution for 12 h.

Bacterial Strains

Escherichia coli RR1 described previously (16) was used as host strain for the construction of the cDNA library.

RNA and DNA Isolation

Total and poly(A)$^+$ RNA were isolated from alfalfa seedlings essentially as described elsewhere (17). High mol wt alfalfa DNA was isolated from purified nuclei using CsCl/ethidium bromide gradients (24). Plasmid DNA was isolated from *E. coli* cells by lysis with Triton X-100 followed by CsCl/EtBr gradient centrifugation (23).

cDNA Synthesis and Cloning

Poly(A)$^+$ RNA was isolated from alfalfa seedlings which had been cold-acclimated for 14 days as described previously (17). ds-cDNA was synthesized from this RNA essentially by the method of Gubler and Hoffman (7). The ds cDNAs were tailed with dCTP (25) and ligated to *Pst*I cut, G-tailed pBR322. The ligation products were transformed into *E. coli* strain RR1 (16). cDNA reaction yields were calculated by including [α-^{32}P]dCTP in the first strand reaction and determining the incorporation at the end of each reaction. Typically from 5 μg poly(A)$^+$ RNA 1.9 μg ss cDNA was synthesized which was quantitatively reproduced in synthesizing ds cDNA.

Screening of cDNA Library and Southern Hybridization

Preliminary screening of the library was carried out following the principle of differential hybridization as described elsewhere (6). The recombinant colonies were immobilized on nitrocellulose filters as described (16). The filters were hybridized to radiolabeled ss cDNA probes prepared from poly(A)$^+$ RNA of nonacclimated or acclimated seedlings. To confirm the results of the primary screening, ^{32}P-labeled cDNA probes were hybridized to replica filters containing equal amounts of *Pst*I-digested plasmid DNAs of the positive clones. Unique sequence species were determined by hybridization of colony blots with cDNA inserts radiolabeled to high specific activity with [α-^{32}P]dCTP by nick translation (20). Unincorporated nucleotides were separated from labeled DNA on Sephadex G-50. Southern blot hybridization and autoradiography were carried out as described (22). All DNA-DNA hybridizations were performed at 65°C in 6× SSC buffer/2% BSA/0.2% polyvinyl pyrrolidone/0.2% Ficoll/0.1% SDS containing denatured calf thymus DNA at 50 μg/ml and the radiolabeled probe at 25 to 100 ng/mL reaction. Following hybridization, the filters were washed twice, 15 min each time, with 2× SSC and 0.1% SDS at room temperature, once for 30 min at 60°C with the same and finally for 30 min with 0.2× SSC and 0.1% SDS at 60°C.

RNA Blotting and Hybridization

Poly(A)$^+$ RNA was separated by electrophoresis through a 2% agarose gel containing 2.2 M formaldehyde (15). The separated RNAs were blotted onto nitrocellulose (S&S). RNA dot blots were prepared by spotting the measured amount of total RNA onto dry nitrocellulose (presoaked in 20× SSC), baked for 2 h at 80°C under vacuum. The RNA gel-blot and RNA dot-blot filters were hybridized as for Southern blot hybridizations except that 0.01% diethylpyrocarbonate was added to the hybridization buffer and the hybridization reaction was carried out at 65°C.

RESULTS

Differential Gene Expression during Cold-Acclimation

A cDNA library was prepared in pBR322 from poly(A)$^+$ RNA from the cold-acclimated seedlings of cultivar Anik. A preliminary differential screening of 3500 Tet^r Amp^s colonies was carried out by hybridization of duplicate nitrocellulose filter replicas of colonies with radiolabeled cDNA probes prepared from poly(A)$^+$ RNA of either nonacclimated or acclimated seedlings. About 17% of the library hybridized to the probe from cold-acclimated seedlings, whereas 13% of clones hybridized well to the nonacclimated probe and were set aside. From the clones showing either positive or negative regulation during cold acclimation, 18 were randomly chosen for further analysis. DNA from each of these clones was restriction-digested with *Pst*I, electrophoresed on agarose gels, blotted on nitrocellulose, and hybridized with radiolabeled, ss-cDNA probes made from either nonacclimated or acclimated mRNA. It was found (results not shown) that inserts of some clones showed stronger hybridization with the ss-cDNAs from nonacclimated seedlings compared to the acclimated while those of many others specifically hybridized to the ss-cDNAs from acclimated seedlings. Furthermore, there were several clones the inserts of which hybridized to the cDNAs from both nonacclimated and acclimated seedlings but hybridized to a greater extent to those from the acclimated seedlings. It was, therefore, concluded that cold acclimation is associated with altered gene expression. The clones bearing the inserts which hybridize specifically to the ss-cDNAs from acclimated seedlings are referred to as CAS clones and their inserts as CAS sequences. A summary of characteristics of three CAS clones is presented in Table I.

RNA-Blot Hybridization with Three Different CAS Clones

In order to select CAS cDNA clones representing different sequences, the interrelationships between these clones were

Table I. *Characterization of Alfalfa CAS cDNA Clones*

Clone	Insert Size	Fraction of Library represented[a]	Transcript Sizes	Genomic *Eco*RI fragments	
				Number	Size
	kb	%	*kb*		*kb*
PSM 784	738	6	1.4, 1.0, 0.4	6	4–11
PSM 2358	861	3.3	1.2, 0.9	7	4.8–12
PSM 2201	720	1.5	0.9	2	5.5–6.1

[a] Based on screening of 3500 colonies.

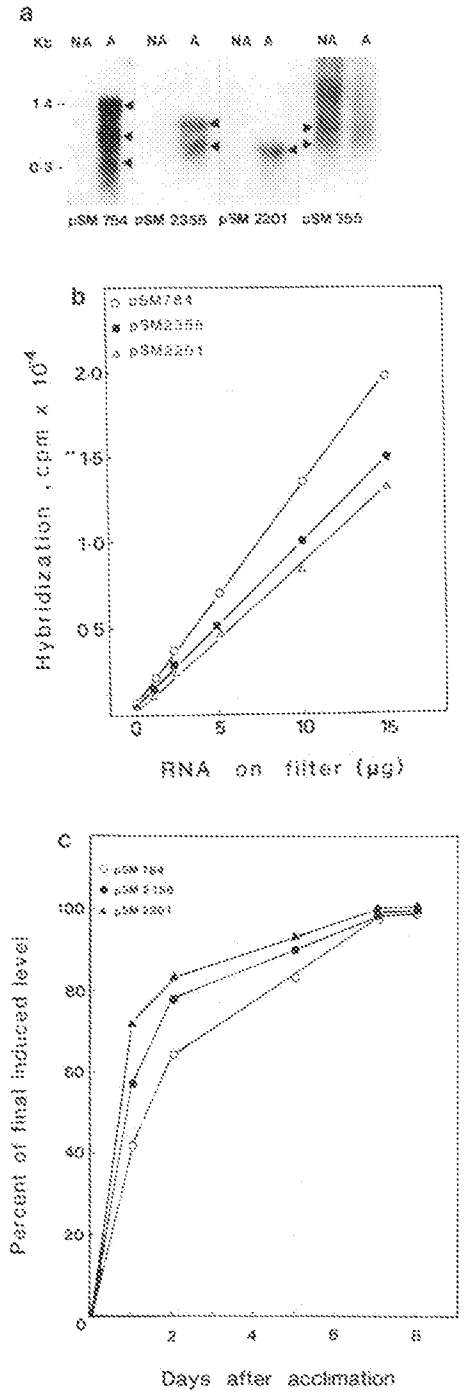

Figure 1. Identification and characterization of three CAS cDNA clones. a, Northern hybridization: 1 μg of Poly(A)$^+$ RNA from nonacclimated (NA) and acclimated (A) seedlings was separated by electrophoresis, blotted onto nitrocellulose and hybridized to nick-translated cDNA inserts of clones mentioned under each set of lanes. Evidence that RNA was not degraded and was equally loaded and transferred to nitrocellulose, was obtained by staining the RNA containing gel with ethidium bromide and observing the gel before and after transfer. The numbers on the left indicate the molecular weight markers (RNA ladder from BRL). b, Determination of relative RNA concentration of cloned sequences. Total RNA was isolated from cold-acclimated and nonacclimated seedlings and as measured by spectrophotometric analysis was fixed to filters. The filters were hybridized to various probes at approximately equal DNA concentrations. The fraction of probe hybridizing to RNA spots was determined by scintillation counting and these values are plotted against μg RNA in the spot. The relative concentrations of transcripts corresponding to various clones are indicated by the slopes of respective curves. c, Time-course of accumulation of the transcripts corresponding to cDNA clones pSM784, pSM2358, and pSM2201 during acclimation period. Ten μg of total RNA isolated from seedlings at 0, 1, 2, 5, 7, and 8 d of acclimation was dot-blotted in triplicate onto a nitrocellulose filter and hybridized to the nick-translated cDNA inserts. The hybridization of each dot was quantified by liquid scintillation spectrometry. The values are means of three replicates (SE $\leq$ 10% of the mean).

determined by cross-hybridization. Such experiments (data not presented) indicated the existence of at least three unique species of sequences. Three cDNA clones—pSM784, pSM2201 and pSM2358—each representing one of the three different sequences, were selected for further study. Their CAS expression was confirmed by Northern hybridization where poly(A)$^+$ RNA from either nonacclimated or acclimated seedlings was electrophoresed on agarose gels, blotted onto nitrocellulose and allowed to hybridize with nick-translated inserts from each of the 3 clones. The autoradiographs of these Northern hybridizations are shown in Figure 1a. It is clear that none of the clones hybridizes to transcripts from nonacclimated seedlings. In each case the hybridization is specific to transcripts from acclimated seedlings. Insert probe from pSM784 hybridizes to two prominent and one faint transcript bands. Probes from pSM2358 and pSM2201 hybridize to two transcript bands and one transcript band respectively. One of these filters (pSM2201) was washed to remove the probe and was allowed to hybridize with the insert probe from the cDNA clone pSM355 which appeared to express equally in nonacclimated and acclimated tissues and hybridized to two transcript bands from nonacclimated and cold-acclimated RNA lanes.

Relative Abundance and Time-Course of Accumulation of CAS Transcripts

The relative abundance, in the acclimated tissue, of transcripts corresponding to each of the 3 clones was determined by quantitative RNA dot-hybridization carried out as described elsewhere (14). The data on partial sequencing of the cDNA inserts presently available show that they have similar guanine-cytosine contents (data not presented). Since the cDNA inserts are of similar lengths and could be radiolabeled with about equal specific activity, the relative abundance of transcripts corresponding to these inserts could be determined by RNA dot-hybridization. To do this, radioactivity hybridized was plotted against the quantity of RNA used. Results are shown in Figure 1b. The relative abundance of transcripts corresponding to each clone is indicated by the slope of the respective curve. It is clear that transcripts homologous to pSM784 are the most abundant, followed in order by pSM2358 and pSM2201. The time-course of accumulation of transcripts homologous to each clone was determined during cold acclimation, by RNA dot-blot hybridization (Fig. 1c). The accumulation of transcripts is rapid during the first day of acclimation but slows down thereafter. It should be noted that transcripts homologous to clones pSM784,

pSM2201 and pSM2358 reach their maximum levels at 7 d of cold-acclimation when a steady state level is achieved.

Restriction Analysis of Alfalfa Chromosomal DNA

In order to determine the possible causes for different relative abundances of transcripts homologous to different clones, relative copy numbers for the corresponding genes were investigated. This was done by Southern hybridization of nick-translated insert probes to equal amounts of nuclear DNA restriction-digested with *Eco*RI. Results are shown in Figure 2. It is clear that there are several *Eco*RI fragments which hybridize to the probe from pSM784. While the pSM 2358 probe hybridizes to two major and several weaker bands the pSM2201 probe hybridizes to only two bands with medium intensity. Similar results were obtained when *Bam*H1 restricted genomic DNA was used (results not presented). No *Eco*RI restriction site was found in the cDNA clones pSM784, pSM2201, and pSM2358. DNA isolated from nonacclimated seedlings also gave similar results.

Specificity of CAS Gene Expression

The possibility was examined that cold-acclimation-induced transcription may reflect a general response of the seedlings to various stresses. RNA dot-blot hybridizations with radiolabeled insert probes from the three CAS clones were carried out. RNA from seedlings subjected to cold-acclimation, ABA treatment, heat shock, water stress, or wounding was used. The results are shown in Figure 3. It is noteworthy that the transcription of genes corresponding to clones pSM784, pSM2358, and pSM2201 is highly specific to cold-acclimation. Nonacclimated, control plants show a level of transcripts which is only a few percent of that in the cold-acclimated plants. Other environmental stress such as water stress, heat shock, wounding, or ABA treatment do not cause appreciable increase in transcript levels. Thus, it is concluded that expression of the genes corresponding to clones pSM784, pSM2358, and pSM2201 is highly specific to cold acclimation and is not a general stress response.

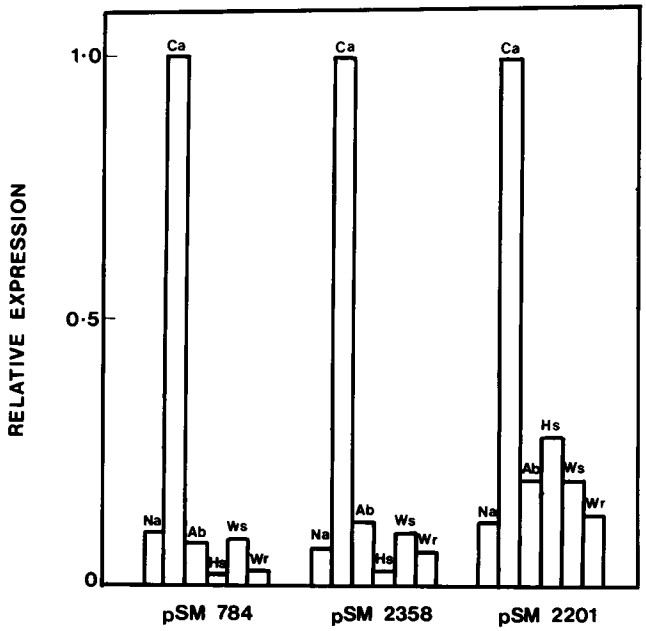

Figure 3. Determination of specificity of CAS transcripts. Total RNA isolated from nonacclimated seedlings (Na), and from seedlings subjected to cold-acclimation (Ca), abscisic acid treatment (Ab), heat shock (Hs), water stress (Ws) and wounding (Wr). Ten μg RNA from each were dot-blotted in triplicate onto nitrocellulose filters and hybridized to nick translated cDNA inserts of clones pSM784, pSM2358, and pSM2201. The hybridization of each dot was quantified by liquid scintillation spectrometry. Values are means of three replicates (SE $\leq$ 10%). The hybridization counts from each treatment expressed in relation to hybridization observed in cold-acclimated seedlings taken as 1.0.

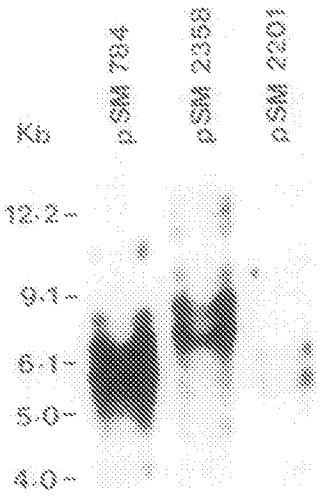

Figure 2. Hybridization of alfalfa genomic DNA fragments with CAS cDNA clones. Ten μg of nuclear DNA was isolated, digested with EcoRI, electrophoresed, blotted onto nitrocellulose and hybridized to nick-translated cDNA inserts (100 ng/ml) from pSM784, pSM2358, and pSM2201. Following hybridization, the filters were washed as described in "Materials and Methods" except that the final wash was for 30 min with 0.5 × SSC and 0.1% SDS at 60°C. The filters were then autoradiographed for 12 h with intensifying screens. The numbers on the left margin indicate mol wt markers (1-kb ladder) in kb pairs.

CAS-Gene Expression Related to Freezing-Tolerance

The relationship between cold-acclimation-induced gene expression and freezing-tolerance of several alfalfa cultivars was examined. RNA dot-blot hybridization with radiolabeled insert probes was carried out using RNA from cold-acclimated seedlings of four different cultivars of alfalfa representing three cultivated species. Data presented in Table II indicate that cold-acclimation induces the expression of genes corresponding to each of the 3 clones, in each cultivar. However, the level of expression is different in different cultivars. Thus, the levels of expression of the genes corresponding to pSM784 in cultivars Iroquois, Algonquin, and Trek are, respectively, 33%, 24%, and 10% of that in Anik. A similar trend is shown by the expression of the genes corresponding to pSM2358 and pSM2201. The level of expression in nonacclimated seedlings was similar in all cultivars and was only 3 to 4% for pSM784,

Table II. *Expression of CAS Sequences and Freezing-Tolerance of Four Alfalfa Cultivars*

Total RNA from various cultivars after 2, 8, and 15 d of cold-acclimation as well as from nonacclimated seedlings was isolated, dotted in triplicate onto nitrocellulose and allowed to hybridize with nick-transplated insert probes of pSM784, pSM2358, and pSM2201. The hybridized dots were quantified by liquid scintillation spectrometry. The values indicate the sum total of hybridization (means of three replicates, SE did not exceed 10%) to 2, 8 and 15 d-acclimated RNAs. The hybridization of nonacclimated RNA in all genotypes was similar and constituted only 3 to 4% of the acclimated Anik control. The values in parenthesis indicate expression of the corresponding sequences as percent of that in Anik.

Alfalfa Cultivars (species)	Relative Expression				LT_{50}
	pSM355 (control)	pSM784	pSM2358	pSM2201	
			cpm × 10		°C
Anik (*M. falcata*)	5.1 (100)	49.2 (100)	8.2 (100)	33.4 (100)	−14.6
Iroquois (*M. media*)	5.2 (102)	16.5 (33)	3.4 (41)	17.7 (53)	−11.8
ALgonquin (*M.* media)	5.5 (108)	12.1 (24)	3.0 (36)	14.36 (43)	−11.5
Trek (*M. sativa*)	4.9 (96)	5.4 (10)	1.3 (15)	7.6 (23)	−9.7
r[a]	−0.055	0.968	0.987	0.993	

[a] The correlation coefficient (r) for each clone indicates the correlation between the level of expression of the cloned sequence and LT_{50} in different cultivars.

pSM2358, and pSM2201 of that in cold-acclimated Anik seedlings. It is of significance to note that the relative level of expression of CAS genes shows a high, positive correlation with freezing-tolerance of the cultivars as determined by LT_{50} values. Thus, the cultivar Trek with an LT_{50} value of −9.7°C is the least freezing-tolerant of the cultivars and shows the lowest level of expression of CAS genes. The cultivar Anik, on the other hand, is the most freezing-tolerant with an LT_{50} value of −14.6°C and shows the highest level of expression of CAS genes. Moreover, cultivars Algonquin and Iroquois, which exhibit intermediate levels of freezing tolerance, show intermediate levels of expression of these CAS sequences.

DISCUSSION

The present study demonstrates that (a) cold-acclimation of alfalfa is accompanied by specific expression of several genes, and (b) the level of expression of these genes, as measured by transcript abundance, is positively correlated with the degree of freezing-tolerance of several alfalfa cultivars.

The previously reported changes in enzyme activities (5, 10, 13, 21) and patterns of protein synthesis (8, 9, 17, 18) during cold-acclimation suggested the possibility that cold-acclimation involves altered gene expression. In order to investigate the molecular genetic basis of freezing tolerance in the present study, we have constructed a cDNA library and identified CAS cDNA sequences. The frequency at which a particular clone is present in the library matches closely with relative abundance of the corresponding RNA suggesting that the library properly reflects the composition of the poly(A)$^+$ RNA population from the cold-acclimated seedlings. Many of the mRNAs in nonacclimated seedlings appear to remain detectable in their expression during cold-acclimation. This is contrary to their response to heat shock (12). Probably these mRNAs represent the house-keeping genes which may be down-regulated due to low temperature but not switched off.

The cDNA clones isolated and examined in the present study appear to be specific to cold-acclimation. The relative abundance of the transcripts homologous to these clones is different and is consistent with the Northern hybridization pattern in that the relatively more abundant clones hybridize to more than one transcript bands. This may be due, at least partly, to the varying copy number of the genes as suggested by the results shown in Figure 2. This conclusion needs to be tempered with caution, however, since we presently lack information on the existence of repeated elements which may also determine the hybridization pattern. Absence of detectable mRNAs corresponding to these CAS clones in seedlings prior to acclimation and their rapid accumulation during cold-acclimation is suggestive of regulation of these genes at the transcriptional level. Furthermore, since transcripts corresponding to the three cDNA clones appear to accumulate during approximately the same time period, the corresponding genes may be regulated in a co-ordinated fashion.

The three clones differ in their pattern of southern hybridization with the *Eco*RI-restricted alfalfa genomic DNA. Since pSM784 shows major hybridization with several bands it is likely that the mRNA corresponding to this clone is encoded by the members of a multigene family or by members of different gene families which share some common sequences. The weak hybridization bands observed with probes from pSM2358 or pSM2201 may not represent hybridization with coding sequences. They may represent 3′-untranslated sequences dispersed throughout the genome. Thus the mRNAs corresponding to pSM2358 and pSM2201 may be encoded by low-copy-number genes.

The observation that ABA and environmental stresses such as heat shock, water stress, and wounding do not induce CAS mRNAs suggests that the cold-acclimation-induced expression of the cloned sequences is specific to cold-acclimation rather than being a general response to environmental stresses. Detection of these CAS mRNAs may explain why ABA alone promotes only a submaximal level of freezing tolerance in alfalfa seedlings (19).

One of the most significant findings of the present study is that the level of the cold-acclimation-induced transcript accumulation in several cultivars of alfalfa is positively correlated with the freezing-tolerance of the cultivars. It should be

noted that all cultivars used in this study possess genes homologous to CAS cDNA clones and yet show different levels of their transcript accumulation and correspondingly different levels of freezing-tolerance. It has been suggested (4) that it is not just the presence or absence of these genes but also the level of their expression which may determine the freezing tolerance of alfalfa cultivars. The reasons for the varietal differences in the level of expression are not known. If the regulation of this expression occurs at the level of transcription, as is likely, then the possible reasons may include differences in (a) copy number of the genes involved; (b) promoter sequences resulting in different transcription efficiencies; and (c) the availability and/or efficiency of promoter-binding proteins and other transcriptional factors. It needs to be pointed out, however, that our data do not allow us to rule out the possibility that the observed changes in transcript levels may arise, at least in part, from changes in mRNA processing and stability.

The results of the experiments reported here suggest that the cloned sequences may be involved in the development of freezing-tolerance in alfalfa. We have previously demonstrated the cold-acclimation-specific synthesis of several polypeptides (17). It is possible that the mRNA accumulation corresponding to the cloned sequences shown here is related to the synthesis of those polypeptides. Isolation and sequencing of the corresponding genes are necessary in order to examine the regulation of their expression and the roles they play in the development of freezing-tolerance. In order to establish the causal relationship between the expression of the cloned sequences and the acquisition of freezing tolerance several of the genes will have to be isolated, transferred to sensitive cultivars and development of freezing tolerance will have to be demonstrated. Since alfalfa is tetraploid with a complex genome, and since freezing tolerance is likely to be determined by many genes progress is likely to be slow. Nevertheless, in view of the correlation observed between the expression of the cloned sequences and the degree of freezing-tolerance, it is tempting to suggest the use of the cloned probes in assessing alfalfa breeding lines for their potential freezing-tolerance.

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